Original article

The expression of genes involved in the ergosterol biosynthesis pathway in *Candida albicans* and *Candida dubliniensis* biofilms exposed to fluconazole

Silvia Borecká-Melkusová,[1] Gary P. Moran,[2] Derek J. Sullivan,[2] Soňa Kucharíková,[1] Dušan Chorvát Jr[3] and Helena Bujdáková[1]

[1]Department of Microbiology and Virology, Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia, [2]Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental School and Hospital, University of Dublin, Trinity College, Dublin, Ireland and [3]Department of Biophotonics, International Laser Centre, Bratislava, Slovakia

Summary

The expression of the *ERG1*, *ERG3*, *ERG7*, *ERG9*, *ERG11* and *ERG25* genes in response to incubation with fluconazole and biofilm formation was investigated using reverse-transcription PCR and real-time PCR in *Candida albicans* and *Candida dubliniensis* clinical isolates. The viability of biofilm was measured using an 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay and confocal scanning laser microscopy (CSLM). Expression of the *ERG11* gene was found to be low or moderate and it was regulated by fluconazole addition more so than by biofilm formation. Very low or non-detectable expression of *ERG1*, *ERG7* and *ERG25* genes was detected in *C. albicans.* The expression of the *ERG9* increased in the presence of fluconazole in some isolates. Following incubation with fluconazole, formation of biofilm by *C. dubliniensis* was coupled with up-regulation of the *ERG3* and *ERG25* genes as have been observed previously in *C. albicans*. Planktonic cells of both *Candida* species released from biofilm displayed similar resistance mechanisms to fluconazole like attached cells. The XTT reduction assay and CSLM revealed that although incubation with fluconazole decreased the biofilm thickness, these were still comprised metabolically active cells able to disseminate and produce biofilm. Our data indicate that biofilm represents a highly adapted community reflecting the individuality of clinical isolates.

Key words: *Candida albicans*, *Candida dubliniensis*, biofilm, resistance.

Introduction

In the aetiology of disseminated infections, the formation of biofilms by pathogenic *Candida* species is often critical.[1–3] A wide range of biomaterials used in clinical practice enables colonisation and subsequent formation of biofilm by *Candida* spp. *Candida albicans* is predominantly isolated from clinical material related to biofilm-associated infections.[4–7] *Candida dubliniensis* is phylogenetically closely related to *C. albicans* and these two species share many virulence factors. Moreover, both are able to form biofilms with similar structural heterogeneity, typical microcolony and water channel architecture.[8,9]

The process of biofilm formation by *C. albicans* involves several steps. Chandra *et al.* described three temporal developmental phases: early (0–11 h), intermediate (12–30 h), and mature (38–72 h).[10] Some recent reports suggested the presence of possible fourth dispersal stage that allows production and release of less adherent daughter cells from mature biofilm.[11,12]

In general, biofilm-associated infections are frequently refractory to conventional antibiotic therapy because of increased resistance to antimicrobial agents.[5,13–15] *Candida albicans* biofilms have been shown to be resistant to a

Correspondence: Silvia Borecká-Melkusová, Department of Microbiology and Virology, Faculty of Natural Sciences, Comenius University, Mlynská dolina B-2, 842 15 Bratislava, Slovakia.
Tel.: +421 2 6029 6482. Fax: +421 2 6029 6480.
E-mail: borecka@fns.uniba.sk

Accepted for publication 6 May 2008

variety of azoles including fluconazole, voriconazole, miconazole, itraconazole, ketoconazole, the antiseptic chlorhexidine, antimetabolite drug flucytosine, and the polyenes nystatin and amphotericin B.[4,10,15–19] Resistance to antifungals is predicted partially because of the formation of a stratified biofilm and production of the extracellular matrix that decrease the diffusion of antifungals into the biofilm.[20,21] Drug resistance occurring during the initial stage of biofilm development is mediated largely by the efflux pumps Mdr1, Cdr1 and Cdr2.[22,23] The increase of drug resistance to fluconazole and amphotericin B during biofilm maturation seems to be associated with a significant decrease in the total ergosterol content as well as with changes in the abundance of other sterols.[23] This fact has been supported by observed changes in the expression of some *ERG* genes involved in ergosterol biosynthesis during maturation of biofilms in comparison with planktonic cells.[24] The major role in the mentioned pathway is played by the enzyme cytochrome P450 lanosterol 14α-demethylase (also referred to as CYP51 and Erg11p) encoded by the *ERG11* gene.[25] Mutations or changes in *ERG11* gene expression as well as changes in other *ERG* genes such *ERG1* (encodes squalene epoxidase), *ERG3* (encodes Δ5,6-desaturase), *ERG7* (encodes squalene cyclase), *ERG9* (encodes squalene synthase) or *ERG25* (encodes C-4 methyl sterol oxidase) play important role in the resistance of planktonic cells to different antifungal agents.[26–29] Although there are many papers discussing the changes in cell physiology and expression of different genes during biofilm formation,[24,26,30,31] *ERG* gene expression during formation of biofilm exposed to azole derivatives has not been investigated. Additionally, while previous studies of biofilm development and resistance mechanisms have been focused predominantly on *C. albicans*, relatively little information is known concerning *C. dubliniensis* biofilm formation.

The purpose of our study was to investigate the effect of fluconazole on biofilm viability and to investigate the potential role of changes in the expression of *ERG* genes (*ERG1*, *ERG3*, *ERG7*, *ERG9*, *ERG11* and *ERG25*) during the early (1.5 and 6 h), intermediate (24 h) and mature (48 h) phases of biofilm formation by *C. albicans* and *C. dubliniensis* clinical isolates.

Materials and methods

C. albicans and *C. dubliniensis* clinical isolates

The three clinical *C. albicans* isolates (1395, 1173 and 47604, originated from Slovak hospitals) and one *C. dubliniensis* clinical isolate (NIH 0492, kindly provided by Prof. Y. Mikami) studied were recovered from immunocompromised patients (stool samples from patients with allergy – *C. albicans* 1395 and 1173, AIDS – *C. albicans* 47604 and a blood sample from patient with cancer – *C. dubliniensis* NIH 0492). The clinical isolates were selected for their reduced susceptibility to fluconazole (MIC_{95} > 64 mg l^{-1}).[32] Fluconazole-susceptible *C. albicans* SC5314[33] and *C. dubliniensis* CBS 7987 (Centraal Bureau voor Schimmelcultures, Delft, The Netherlands) were used as reference strains. Isolates were maintained in glycerol/YPD (2% glucose, 1% yeast extract, 2% bactopeptone) at −80 °C.

Minimal inhibitory concentration (MIC) determinations

The MIC_{95} values (drug concentration that inhibited growth by 95% compared with the growth of positive controls) for fluconazole (Pfizer, Zürich, Switzerland) in tested strains were published previously (MIC_{95} > 64 mg l^{-1}).[32] The MIC_{95} for reference strains *C. albicans* SC5314 and *C. dubliniensis* CBS 7987 were determined by the broth microdilution method according to the CLSI M27-A2 reference method in RPMI 1640 medium [Applichem GmbH, Darmstadt, Germany; buffered with 0.165 mol l^{-1} 3-(*N*-morpholino) propanesulfonic acid, pH 7.0] in this work.[34] The $0.5 \times MIC_{95}$ values for fluconazole were used in subsequent experiments. Strains were able to grow at these concentrations without significant reduction (they reached OD_{600} = 1 in overnight culture).

Preparation of yeast suspensions for the biofilm formation

Yeast strains were cultivated on Sabouraud dextrose agar (Biomark Laboratories, Puna, India) at 28 °C for 24 h before use. For each strain, a large loop of cells was transferred to the 200 ml of Yeast Nitrogen Base broth (YNB) (Sigma-Aldrich, Taukirchen, Germany) containing 0.9% of D-glucose and supplemented with or without subinhibitory concentrations of fluconazole. After overnight incubation at 37 °C, the cells were centrifuged and washed twice with 30 ml of phosphate-buffered saline (PBS), vortexed and centrifuged at 5000 *g* for 5 min. The cells were then resuspended in 50 ml of YNB broth containing 0.9% of D-glucose and supplemented with or without subinhibitory concentrations of fluconazole. Every sample was adjusted to a final OD_{600} = 1.0 with YNB broth supplemented with or without antifungal agent. These cell suspensions were then used for biofilm formation.

The microtitre plate model of biofilm formation and the effect of fluconazole on biofilm formation

A standardised method for biofilm formation using polystyrene 96-well plates (flat bottom; DispoLab, Brno, Czech Republic) was used.[35] For biofilm growth, YNB broth without/with fluconazole was used. After 48-h period, biofilm formation was quantified using the 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay.[35] The XTT stock solution was prepared at 1 g l^{-1}. A colorimetric change was measured in a microplate reader (MRX Microtitre plate absorbance reader; Dynex Technologies, Chantilly, VA, USA) at 490 nm. Three repeats were performed for each strain.

Slide model of biofilm formation for CSLM

Biofilms were formed on 25-mm diameter highly adherent polyethylene coverslips (Sarstedt, Nümbrecht, Germany) as described previously.[4] Coverslips were immersed in 20 ml of a standardised cell suspension (OD_{600} = 1.0) prepared as described above. Petri dishes (Sarstedt) with prepared samples were incubated at 37 °C for 90 min. After the adhesion period, supernatants including planktonic cells were discarded and Petri dishes together with coverslips were washed twice with 20 ml of PBS. Twenty millilitres of fresh YNB broth supplemented with or without subinhibitory concentrations of fluconazole was added to each Petri dish and incubated at 37 °C for 48 h for biofilm growth. Following biofilm formation, coverslips were gently washed with PBS and used for confocal scanning laser microscopy (CSLM).

Confocal scanning laser microscopy

Mature biofilms formed on polyethylene coverslips were transferred to a new Petri dish and 20 μl of tetramethyl rhodamine methyl ester, perchlorate (TMRM; Invitrogen, Carlsbad, CA, USA; excitation wavelength 549 nm, emission wavelength 573 nm), diluted in distilled deionised water to final concentration of 5 μmol l^{-1}, was applied onto each coverslip. Stained biofilms were observed with a LSM 510 META confocal scanning laser microscope mounted on Axiovert 200 M inverted microscope (both from Carl Zeiss, Jena, Germany). A 543-nm line of He–Ne laser line for excitation, 545 nm dichroic mirror and 565–615 nm band-pass emission filter for fluorescence detection were employed. A 20 × /0.75 PlanApochromat dry objective was used, with the confocal pinhole opening of 1 airy unit. The half-width of point-spread function in Z direction was 1.8–2 μm (in 500–600 nm wavelength range); therefore, we used 0.9 μm Z-axis sampling in three dimensional data recording. For each image, 153 × 153 μm area was scanned with resolution of 512 × 512 pixels, 16 × line averaging and 8-bit quantisation. Images were further processed in the LSM IMAGE EXAMINER software (Carl Zeiss). Biofilm images were either displayed individually as 2-D plots or reconstructed in three-dimensional (3-D) projections. Vertical (*xz*) sections or side views of the 3-D reconstructed images were used to determine biofilm thickness and architecture. The thickness was estimated from outer edges of the area, where TMRM signal gain intensity was above half of its maximum.

Preparation of biofilm in polystyrene Petri dishes for qualitative sterol analysis, reverse-transcription PCR and quantitative real-time PCR analysis

Modification of the standardised method for biofilm formation in microtitre plate was used.[35] For each strain, 30 ml of the suspension (OD_{600} = 1.0) was inoculated into four polystyrene Petri dishes (90-mm diameter; Sarstedt). The plates were incubated at 37 °C for 90 min to allow the adhesion of cells to polystyrene surface. Planktonic cells from the first Petri dish were collected, the adhered cells were gently washed twice with 20 ml of sterile PBS and both planktonic and adhered cells were used for RNA isolation. Supernatants including planktonic cells and liquid medium from the next three Petri dishes were discarded and adhered cells were gently washed twice with 20 ml of sterile PBS. For biofilm growth, 30 ml of fresh YNB broth containing 0.9% of D-glucose supplemented with or without fluconazole was added to each Petri dish. The samples were cultivated for 6, 24 or 48 h at 37 °C. At these time points, planktonic cells were collected and formed biofilms were washed with 20 ml of sterile PBS buffer. Biofilm cultures formed in the Petri dishes were scraped using cell scraper (Becton Dickinson, Le Pont-de-Claix, France), resuspended in sterile PBS and directly used for qualitative sterol analysis and RNA isolation or flash-frozen in liquid nitrogen and stored at −80 °C until use.

Qualitative sterol analysis

Total sterols extracted from both planktonic cells and biofilm were estimated using UV spectrophotometric analysis as described by Moran *et al.* [36].

Reverse transcription-PCR (RT-PCR) analysis

Genomic DNA was extracted from each isolate according to Xu *et al.* [37]. RNA for RT-PCR analysis was extracted from *C. albicans* and *C. dubliniensis* planktonic cells and biofilm using RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Aliquots of RNA (2 μg) were treated with RNase-free DNase I (TaKaRa, Otsu, Japan) and stored at −80 °C until use. The TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) were used to synthesise cDNA according to the manufacture's instructions. Random hexamers were used to prime the cDNA synthesis reaction. Subsequent PCR was performed with the primers amplifying genes *ACT1*, *ERG1*, *CaERG3*, *ERG7*, *ERG9*, *ERG11* and *ERG25* according to Henry *et al.* [27]. The primer pair CdERG3F-CdERG3R was derived from the primer sequence used by Pinjon *et al.* [38]. A parallel reaction with DNase treated RNA was performed to confirm that the PCR product was derived from cDNA rather than genomic DNA contamination. A reaction including *C. albicans* or *C. dubliniensis* genomic DNA was used as a positive control for the PCR reaction. PCR was performed by initial denaturation at 94 °C for 3 min, followed by 28 cycles at 94 °C for 1 min, 51 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were analysed by 1.5% agarose gel and visualised by ethidium bromide staining. Relative expression of individual genes was estimated by comparison with *ACT1* gene expression as follows: weak expression (the intensity of respective band was significantly lower than the intensity of those of *ACT1*); m – moderate expression (comparable with those of *ACT1*); s – strong expression (the intensity of respective band was significantly stronger than the intensity of those of *ACT1*).

Quantitative real-time PCR analysis

Real-time PCR was used for analysis of *ERG1*, *ERG25* and *TEF1* gene expression during biofilm formation in *C. albicans* SC5314 and *C. dubliniensis* NIH 0492. RNA was extracted from both planktonic cells and biofilm using TRI-Reagent (Sigma-Aldrich) as described by Stokes *et al.* [39].

Aliquots of RNA (500 ng) were treated with RNase-free RQ1 DNase (Promega, Madison, WI, USA) and cDNA was synthesised with Superscript II reverse transcriptase (Gibco BRL Life Technologies, Rockville, MD, USA) following the manufacturer's instructions. For an internal mRNA control and for detection of genomic DNA, the primers specific for the *EFB1* gene of *C. albicans*, containing an intron of 365 bp were used (Table 1).[40] Furthermore, the absence of genomic DNA contamination was also verified by performing PCR amplification of RNA samples lacking reverse transcriptase; so PCR products were not detected in these control reactions. The control PCR was carried out using Go Taq® Flexi DNA polymerase (Promega).

Real-time PCR was carried out on an ABI Prism 7000 Sequence Detection system (Applied Biosystems) with ABI PRISM 7000 SDS Software using 2 × QuantiTect™ SYBR Green PCR Master Mix (Qiagen) as described by Moran *et al.* [41].

The change in fluorescence of SYBR Green I dye in every cycle was monitored by the software SEQUENCE DETECTOR 1.7 provided with the ABI Prism 7700 system (Applied Biosystems) and the threshold cycle (C_T) above the background for each reaction was calculated. For data analyses, C_T values were exported into a Microsoft Excel Worksheet for further statistical analyses. The average C_T value of housekeeping gene (*TEF1*; gene for elongation factor 1-alpha)[42] was subtracted from the average C_T value of tested genes

Table 1 Primers used for quantitative real-time PCR analysis

Gene	Primer name	GenBank accession no.	Orientation[1]	Sequence (5′ to 3′)	PCR amplicon size (bp)
EFB1	EF1F	X96517	F	ATTGAACGAATTCTTGGCTGAC	cDNA-554
	EF1R		R	CATCTTCTTCAACAGCAGCTTG	DNA-919
TEF1	QRTTEF1F	M29934	F	CCACTGAAGTCAAGTCCGTTGA	51
	QRTTEF1R		R	CACCTTCAGCCAATTGTTCGT	
CaERG1	ERG1F	U69674	F	ACTAATGTTCCACCATTGGCTCT	84
	ERG1R		R	CACATGACCTTTGCCCTTAGCT	
CdERG1[2]	CdERG1F	U69674	F	ACTAATGTTCCACCATTGGTTCT	84
	CdERG1R		R	CACATGACCTTTGCCCTTGGCT	
ERG25	ERG25F	AF051914	F	GCTCATCCAGTTGAAGTTGCC	82
	ERG25R		R	GCAAGTTACCAGTGATAAGACACCA	

[1]F = forward primer; R = reverse primer.

[2]The primer sequence for *CdERG1* was based on the sequence for *CaERG1* gene with simple nucleotide substitution.

ERG1 and *ERG25* to obtain a ΔC_T value. The comparative method ($\Delta\Delta C_T$ method) based on comparing differences in obtained ΔC_T values was used for manifestation of changes in the expression of mentioned genes. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta CT}$. Data for each sample were obtained from three independent experiments.

Results

Susceptibility testing, effect of fluconazole on formation and structure of biofilm and qualitative sterol analysis

The reference strains *C. albicans* SC5314 and *C. dubliniensis* CBS 7987 were susceptible to fluconazole (MIC_{95} = 0.5 mg l^{-1} and MIC_{95} = 0.25 mg l^{-1}, respectively). On the other hand, all clinical isolates exhibited decreased susceptibility to this drug (MIC_{95} > 64 mg l^{-1}).[32]

The XTT reduction assay is the most commonly used method for the determination of biofilm formation. Using this as a marker for biofilm viability, it was confirmed that the presence of fluconazole decreased the ability to form biofilm in all tested *C. albicans* strains to approximately one half (Fig. 1; $P < 0.05$). Similar reduction was confirmed in *C. dublininesis* NIH 0492 ($P < 0.05$). However, no significant effect of fluconazole on biofilm formation was observed in *C. dubliniensis* CBS 7987 ($P > 0.05$).

The structure of mature biofilms formed by *C. albicans* SC5314, *C. albicans* 1173, *C. dubliniensis* CBS 7987 and *C. dubiniensis* NIH 0492 was explored using CSLM (Fig. 2). Biofilm formed by the fluconazole-susceptible reference strain *C. albicans* SC5314 in the absence of fluconazole revealed an approximately 9–10 μm thick biofilm composed of both yeast and hyphal cells. The presence of subinhibitory concentrations of fluconazole decreased the thickness of biofilm by less than half (4–5 μm; $P < 0.05$) and the mature biofilm was composed exclusively of yeast cells. The fluconazole-resistant clinical isolate *C. albicans* 1173 did not form the typical yeast–hyphal mixture in biofilm using method described in Material and methods. The 15–16 μm thick biofilm found to be comprised entirely of yeast cells, but following incubation with fluconazole the biofilm biomass decreased dramatically to negligible values and resulted in the formation of yeast cell aggregated clusters. Biofilm formed by fluconazole-susceptible reference strain *C. dubliniensis* CBS 7987 as well as images of biofilm formed by the fluconazole-resistant clinical isolate *C. dubliniensis* NIH 0492 showed biofilm formed by yeasts covered by

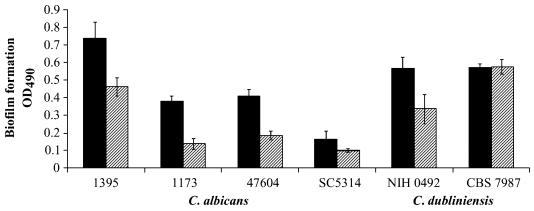

Figure 1 Biofilm formation of *Candida albicans* 1395, *C. albicans* 1173, *C. albicans* 47604, *Candida dubliniensis* NIH0492 and *C. dubliniensis* CBS 7987. The ability to produce biofilm without presence of fluconazole (filled bars) and in the presence of subinhibitory concentrations of fluconazole (hatched bars) was quantified using the XTT-reduction assay. Values are mean of triplicate determinations ± SD of the mean of biofilms formed in 96-well polystyrene microtitre plate.

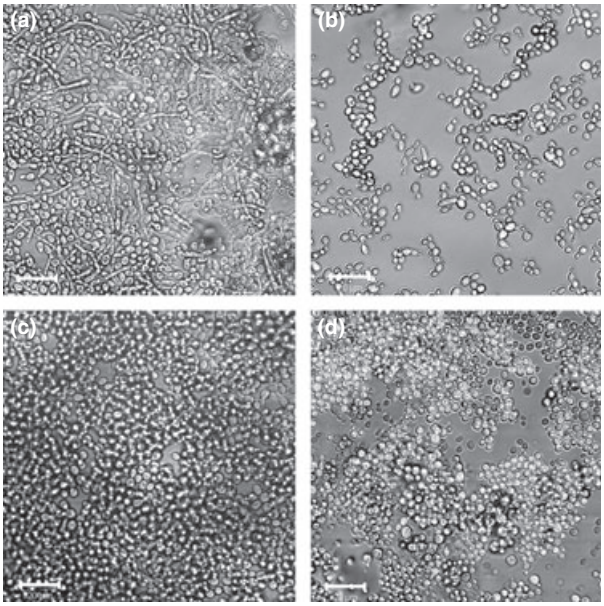

Figure 2 Confocal scanning laser microscopy (CSLM) biofilm images. A CSLM image of a biofilm produced by *C. albicans* SC5314 (a and b) and *C. dubliniensis* CBS 7987 (c and d) on polyethylene coverslips after 48-h cultivation in the absence (a and c) or in the presence of 0.5 × MIC_{95} of fluconazole in the medium (b and d). Scale bar, 20 μm.

extracellular matrix. Incubation with fluconazole caused splitting of the primary contiguous double layer of yeast cells into aggregates formed by metabolically active yeast cells. In a similar fashion to biofilms formed by *C. albicans*, fluconazole decreased the thickness of biofilms formed by *C. dubliniensis* CBS 7987 (from 15–16 to 8–9 μm; $P < 0.05$) and by *C. dubliniensis* NIH 0492 (from 25 to 13–15 μm; $P < 0.05$).

Qualitative analysis of total sterol contents revealed no significant changes in ergosterol profiles during

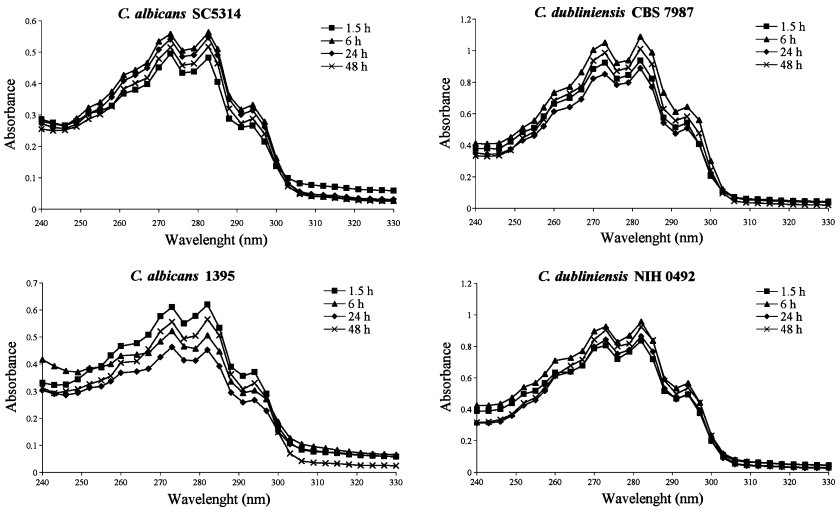

Figure 3 UV spectrophotometric profiles of non-saponifiable lipids from *C. albicans* reference strain SC5314, *C. dubliniensis* reference strain CBS 7987 and *C. albicans* clinical isolate 1395 and *C. dubliniensis* clinical isolate NIH 0492 with reduced susceptibility to fluconazole (MIC_{95} > 64 mg l^{-1}). Sterols were extracted from three biofilm developmental stages: early (1.5 and 6 h), intermediate (24 h), and mature (48 h) as described in Materials and methods.

formation of biofilm. The presence of ergosterol in both *C. albicans* and *C. dubliniensis* was represented by four peaks observed from the first stadium till maturation of biofilm (Fig. 3).

RT-PCR and quantitative real-time PCR analysis

Reverse transcription-PCR was used for screening of the expression of *ERG* genes (*ERG1*, *ERG3*, *ERG7*, *ERG9*, *ERG11* and *ERG25*) involved in the ergosterol biosynthesis pathway of *C. albicans* and *C. dubliniensis* during biofilm formation (at 1.5, 6, 24 and 48 h). The expression of individual genes was estimated by comparison with that shown by the *ACT1* gene (Table 2 and Fig. 4). Results summarised in Table 2 showed that the expression of *ERG1*, *ERG7* and *ERG25* genes in *C. albicans* was observed to be very weak or not detectable with the addition of fluconazole having little or no effect on the expression of these genes. The expression of the *ERG3*, *ERG9* and *ERG11* genes in *C. albicans* varied according to isolate tested. The expression of *ERG3* gene was influenced rather by biofilm formation than fluconazole addition. On the other hand, the presence of fluconazole up-regulated expression of the *ERG9* gene regardless of susceptibility of tested strains. In the fluconazole sensitive reference strain *C. albicans* SC5314, the expression of *ERG11* was found to decrease after incubation with fluconazole, in opposite to fluconazole-resistant isolates *C. albicans* 1173 and *C. albicans* 47604, where incubation with fluconazole increased expression of this gene. Similarly to results obtained in *C. albicans* strains, the expression of both *ERG1* and *ERG7* genes was not observed in *C. dubliniensis*. On the other hand, expression of other tested genes in *C. dubliniensis* was different from those observed in *C. albicans* strains. In fluconazole-resistant *C. dubliniensis* NIH 0492, incubation with fluconazole up-regulated the expression of the genes *ERG25*, *ERG3* and *ERG11*. Moreover, strong up-regulation of the *ERG3* gene affected by fluconazole was found also in fluconazole-sensitive *C. dubliniensis* strain CBS 7987. On the other hand, no expression of the gene *ERG11* was detected in this strain. The expression of the *ERG9* gene in both *C. dubliniensis* isolates tested was weak or moderate and minimally affected by fluconazole. Maturation of biofilm was associated with slightly increased *ERG11* expression, more significant in fluconazole-resistant *C. albicans* isolates than in fluconazole-resistant *C. dubliniensis*.

Based on these results, *C. albicans* SC5314 and *C. dubliniensis* NIH 0492 were selected for detailed analysis of *ERG1* and *ERG25* gene expression using quantitative real-time PCR analysis.

In *C. albicans* strain SC5314, the addition of fluconazole to the medium slightly increased the expression of *ERG1* gene in both planktonic cell population (1.29 ± 0.26 times) and cells forming biofilm (1.27 ± 0.30 times) in comparison with the drug free controls. For clinical isolate *C. dubliniensis* NIH 0492, no amplification of *ERG1* was detected as observed previously by RT-PCR. Subinhibitory concentrations of fluconazole increased the expression of *ERG25* gene in

Table 2 Detection of reverse-transcription PCR (RT-PCR) products during the biofilm formation in *Candida albicans* and *Candida dubliniensis*

		Genes					
Strain	Time (h)	*ERG1* −/+ FLC	*ERG3* −/+ FLC	*ERG7* −/+ FLC	*ERG9* −/+ FLC	*ERG11* −/+ FLC	*ERG25* −/+ FLC
Candida albicans SC5314	1.5	ND/w	ND/m	ND/w	m/m	w/w	ND/w
	6	ND/w	w/w	w/w	m/m	m/w	ND/w
	24	ND/w	m/m	w/w	m/m	m/w	ND/w
	48	ND/ND	m/m	w/w	m/s	m/w	ND/w
Candida albicans 1395	1.5	w/ND	w/w	w/ND	m/s	ND/ND	w/w
	6	w/ND	m/w	w/ND	m/m	ND/ND	w/w
	24	w/ND	m/w	w/ND	m/m	w/w	w/w
	48	w/w	m/m	w/ND	m/s	w/w	w/w
Candida albicans 1173	1.5	ND/ND	ND/w	ND/ND	w/s	ND/m	ND/ND
	6	ND/ND	ND/w	ND/ND	m/s	ND/m	ND/ND
	24	ND/ND	w/w	ND/ND	m/m	ND/w	ND/ND
	48	ND/ND	m/w	w/ND	m/m	m/w	ND/ND
Candida albicans 47604	1.5	w/ND	w/w	w/ND	m/m	w/ND	ND/ND
	6	w/ND	m/w	w/ND	m/s	w/w	w/ND
	24	w/ND	w/w	ND/ND	m/s	w/m	w/ND
	48	w/ND	w/w	ND/ND	m/s	w/m	w/ND
Candida dubliniensis CBS 7987	1.5	ND/ND	m/s	ND/ND	m/w	ND/ND	ND/w
	6	ND/ND	m/s	ND/ND	m/w	ND/ND	ND/w
	24	ND/ND	m/s	ND/ND	m/w	ND/ND	ND/w
	48	ND/ND	m/s	ND/ND	w/w	ND/ND	ND/w
Candida dubliniensis NIH 0492	1.5	ND/ND	m/s	ND/ND	w/m	ND/w	ND/m
	6	ND/ND	m/s	ND/ND	m/m	ND/w	ND/s
	24	ND/ND	m/s	ND/ND	m/m	ND/w	w/s
	48	ND/ND	s/s	ND/ND	m/m	w/w	w/s

Biofilm was produced in medium supplemented without (−) / with (+) fluconazole. Relative expression of individual genes was estimated by comparison with *ACT1* gene expression: ND, not detectable; w, weak expression; m, moderate expression, comparable to those of *ACT1*; s, strong expression; FLC, fluconazole. Data for expressions of individual genes were obtained from two independent experiments.

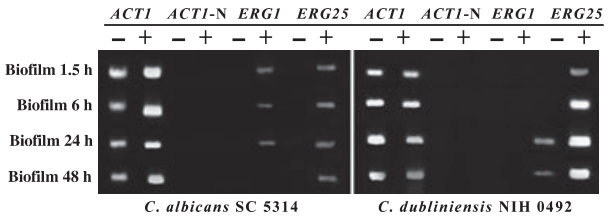

Figure 4 Representative picture of reverse-transcription PCR (RT-PCR) analysis of *C. albicans* SC5314 and *C. dubliniensis* NIH 0492. RT-PCR was used to analyse the relative expression of *ACT1*, *ERG1* and *ERG25* genes in three biofilm developmental stages: early (1.5 and 6 h), intermediate (24 h) and mature (48 h). Equal volumes of DNase treated RNA with primers for actin were amplified in parallel to detect genomic DNA contamination (*ACT1*-N). Following PCR reaction, PCR products were analysed by 1.5% agarose gel and visualised by ethidium bromide staining. Samples were cultivated in Yeast Nitrogen Base broth containing 0.9% of D-glucose and supplemented with (+) or without (−) fluconazole.

C. albicans SC5314 planktonic cells (3.13 ± 0.19 times) and in biofilm (1.65 ± 0.3 times) in comparison with the drug free control. *Candida dubliniensis* NIH 0492 planktonic cells and biofilm showed the highest degree of up-regulation of the *ERG25* gene following exposure to fluconazole in comparison with the drug free controls (3.98 ± 0.15 and 29.77 ± 0.24 times, respectively). The expression of the tested genes in both *C. albicans* and *C. dubliniensis* indicated no significant changes because of formation of biofilm with the exception for *C. dubliniensis* NIH 0492, where the expression of *ERG25* gene increased 1.68 ± 0.5 times in later stages (at 48 h) of biofilm formation in comparison with that observed in the first stage (at 1.5 h).

Parallel analysis focused on the expression of *ERG* genes tested was performed by both methods using planktonic cells. Planktonic cells represented non-attached cells that are released from biofilm during its formation. These cells were collected parallel with biofilm at four time point (at 1.5, 6, 24 and 48 h). The RT-PCR revealed that planktonic cells displayed similar *ERG* gene expression profiles to those observed for biofilm (data not shown). Although using RT-PCR, we did not detect any expression of the *ERG25* gene in *C. albicans* SC5314, the

higher sensitivity of quantitative real-time PCR analysis revealed that planktonic cells without addition of fluconazole exhibited higher expression of the *ERG25* gene in both *C. albicans* SC5314 (6.56 ± 0.38 times) and *C. dubliniensis* NIH 0492 (5.01 ± 0.5 times) strains in comparison with that observed in biofilm cells.

Discussion

Candida spp. can adhere and form biofilm on the surfaces of a wide range of medical devices resulting in reduced susceptibility to antimicrobial agents.[1,2,21,43] *Candida albicans*-like bacterial biofilm-associated resistance has been explained because of multiple mechanisms including drug efflux pumps, extracellular matrix production, decrease of cell metabolism and unique architecture.[44–46] The treatment of *C. albicans* biofilm by antifungals is accompanied by the changes in morphology of biofilm. CSLM in conjunction with labelling by TMRM allowed observation of intact structures containing living cells during biofilm formation. The morphology of biofilm showed to be surface pattern dependent as was already described by Chandra *et al.* [10]. They showed that irregular surface of polymethylmethacrylate allows formation of only 25–30 μm thick biofilm formed mainly by yeast cells. On the other hand, *C. albicans* cells growing on silicone elastomer produce a nearly uniform layer of adherent blastospores, which at maturation are approximately 10–12 μm thick and above this layer is a profuse matrix (at least 450 μm thick) consisting of extracellular material and hyphal elements. The proportions of yeast and hyphal forms depend also on medium flow, aeration, temperature and incubation time.[24] In our study, we used polyethylene coverslips and polystyrene Petri dishes for biofilm formation. Both materials are hydrophobic and allow strong attachment of *Candida* cells to the surface, which is an important step in biofilm formation. Although polystyrene plate is made from material different from that used for intravenous catheters, the adhesion properties of both materials are similar. This is a reason why a standardised method for biofilm formation employing polystyrene 96-well plates is generally accepted by many authors.[18] On the other hand, the advantage of polyethylene coverslips for CSLM is easy manipulation and application of this material in *in vivo* models.[43,44] Nobile *et al.* [47] successfully used the polyethylene catheter in an *in vivo* rat model for biofilm formation. However, polyethylene did not induce the formation of typical yeast-hyphae structure of mature biofilm in all tested strains. In spite of this limitation, the effect of subinhibitory concentrations of fluconazole was notable in all tested strains without any relation to fluconazole susceptibility or resistance. The biofilm thickness as well as density of yeast cell layer was affected. Moreover, fluconazole influenced the property of strain *C. albicans* SC5314 to form hyphae. The morphology of studied biofilms was confirmed by light microscopy of biofilms formed in polystyrene Petri dishes (pictures not shown). Similar observation in fluconazole efficiency on biofilm was published by Bruzual *et al.* [48], who reported inhibition of *C. albicans* biofilm in the presence of fluconazole in the medium regardless of susceptibility of tested strains.

Two major resistance mechanisms have been identified in *C. albicans* and *C. dubliniensis*; expression of multidrug transporters that reduce the drug accumulation[22,23,30,49–52] and alteration of membrane sterol composition represented mainly by changes in ergosterol biosynthesis pathway.[23,26,28,38,53,54]

We investigated the expression of *ERG* genes (*ERG1*, *ERG3*, *ERG7*, *ERG9*, *ERG11* and *ERG25*) employing RT-PCR and real-time PCR in *C. albicans* and *C. dubliniensis* in response to incubation with fluconazole and biofilm formation. Three of the genes examined (*ERG9*, *ERG1* and *ERG7*) encode enzymes that act upstream and two (*ERG25* and *ERG3*) encode enzymes that act downstream of lanosterol demethylase in the ergosterol biosynthesis pathway. The *C. albicans* and *C. dubliniensis* clinical isolates were selected for their reduced susceptibility to fluconazole (MIC_{95} > 64 μg ml^{-1}) and high ability to form biofilm in comparison with standard strain *C. albicans* SC5314.

Up-regulation of *C. albicans ERG11* or *C. dubliniensis ERG11* genes encoding the target enzyme Erg11p for azole antifungal drugs often results in resistance to azole derivatives.[38,52] Henry *et al.* [27] reported that cultivation in the presence of azole drugs increased the expression of *ERG11* gene in planktonic cells, but this induction has not been observed during biofilm formation in standard strains *C. albicans* SC5314 and *C. albicans* CA-1.[24,26] We obtained similar results with *C. albicans* SC5314, but tested clinical isolates reflected variability among strains. The expression of *ERG11* gene was proved low or moderate in comparison with *ACT1* gene and it was influenced by fluconazole addition rather than by biofilm formation. It is of interest that in both *C. dubliniensis* isolates, lower expression of the *ERG11* gene was coupled with an increased level of *ERG3* mRNA. A similar effect was observed in fluconazole resistant mutants of *C. glabrata*.[55] Moreover, the presence of fluconazole strongly influenced the expression of the *ERG3* gene in fluconazole resistant *C. dubliniensis* NIH 0492.

The main difference in the expression of *ERG* genes between *C. albicans* and *C. dubliniensis* was observed in the *ERG25* gene expression. Strong up-regulation of this gene following fluconazole addition was obtained in clinical isolate *C. dubliniensis* NIH 0492. A real-time PCR analysis was in agreement with results from RT-PCR. During biofilm formation, the expression of *ERG25* gene increased, after 48-h biofilm formation, 2.7 times in *C. dubliniensis* NIH 0492 unlike to in *C. albicans* SC5314 in which there was no increase. The up-regulation of *ERG25* and also *ERG2*, *ERG7* and *ERG11* in *C. albicans* following azole treatment has been previously shown to be induced by the transcription factor encoded by the *UPC2* gene. This regulator controls sterol uptake under anaerobic conditions which are encountered in the mature biofilm formed static in a microtitre plate.[24,56] The role of *UPC2* gene in *C. dubliniensis* remains to be further investigated. Moreover, the real-time PCR analysis revealed no expression of *ERG1* gene in *C. dubliniensis* NIH 0492 in contrast to *C. albicans* SC5314 exhibiting slight expression of this gene. A significantly reduced expression of *ERG1* gene in the blastospore subpopulation in *C. albicans* biofilms resistant to amphotericin B has already been reported by Khot *et al.* [26]. Such global up-regulation of multiple genes involved in the ergosterol biosynthetic pathway can shuttle non-ergosterol intermediates into the membrane.[54]

Mukherjee *et al.* [23] in their work revealed by quantitative sterol analysis that total ergosterol level significantly decreased at later stages of biofilm formation in comparison with planktonic cells. Using qualitative sterol analysis in our work, we did not confirm the presented data and we did not observe changes in total sterol profile including ergosterol during formation of biofilm in all tested isolates. On the basis of that information, we hypothesise that mature biofilm communities may represent hypoxic microenvironments with negative effect on the synthesis of new free ergosterol, but with no significant effect on total ergosterol contents as described by Shobayashi *et al.* [57] in *Saccharomyces cerevisiae* laboratory strain X2180-1A.

The comparison of the expression of genes in biofilm and planktonic cells released from the biofilm did not show significant differences and the up-regulation of some *ERG* genes in individual *C. albicans* and *C. dubliniensis* strains correlated with that described for biofilm. Such cells could represent the dispersal stage of biofilm capable of spreading *Candida* infection.[11,12]

Our data suggest that the degree of biofilm formation by clinical isolates is strain-dependent. Previous published information about increased expression of *ERG3* and *ERG25* genes in *C. albicans* during biofim formation was also confirmed in fluconazole-resistant *C. dubliniensis*. Planktonic cells released from biofilm displayed similar mechanisms of resistance to fluconazole as the attached cells. On the other hand, CSLM revealed that although incubation with fluconazole decreased the biofilm thickness and yeast cells aggregated to clusters, these were still formed by metabolically active cells able to disseminate and produce biofilm. This can explain frequent relapses of candidiasis in immunocompromised patients who require prophylactic antifungal therapy. Understanding of the role of different *C. albicans* and *C. dubliniensis* genes in biofilm formation and resistance to antifungal agents could ultimately be helpful for control of *Candida* infections associated with using artificial medical devices.

Acknowledgments

This work was supported by Marie Curie RTN Program MRTN-CT-2004-512481, MVTS 6RP/MRTN-CT-2004-512481 and VEGA 1/4324/07 from Slovak Ministry of Education, Grant No. UK/105/2006 from the Comenius University, Bratislava, Slovak Republic and FEMS Research Fellowship to S. B.-M.

References

1 Douglas LJ. *Candida* biofilms and their role in infection. *Trends Microbiol* 2003; **11**: 30–36.
2 Ramage G, Martínez M, López-Ribot JL. *Candida* biofilms on implanted biomaterials: a clinically significant problem. *FEMS Yeast Res* 2006; **6**: 979–86.
3 Tumbarello M, Posteraro B, Trecarichi EM *et al.* Biofilm production by *Candida* species and inadequate antifungal therapy as predictors of mortality for patients with candidemia. *J Clin Microbiol* 2007; **45**: 1843–50.
4 Chandra J, Mukherjee PK, Leidich SD *et al.* Antifungal resistance of candidal biofilms formed on denture acrylic *in vitro*. *J Dent Res* 2001; **80**: 903–8.
5 Baillie GS, Douglas LJ. *Candida* biofilms and their susceptibility to antifungal agents. *Meth Enzymol* 1999; **310**: 644–56.
6 Ramage G, VandeWalle K, Wickes BL, López-Ribot JL. Characteristics of biofilm formation by *Candida albicans*. *Rev Iberoam Micol* 2001; **18**: 163–70.
7 Ramage G, Saville SP, Thomas DP, López-Ribot JL. *Candida* biofilms: an update. *Eukaryot Cell* 2005; **4**: 633–8.
8 Sullivan D, Coleman D. *Candida dubliniensis*: an emerging opportunistic pathogen. *Curr Top Med Mycol* 1997; **8**: 15–25.
9 Ramage G, Vande Walle K, Wickes BL, López-Ribot JL. Biofilm formation by *Candida dubliniensis*. *J Clin Microbiol* 2001; **39**: 3234–40.

10 Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol* 2001; **183**: 5385–94.
11 Granger BL, Flenniken ML, Davis DA, Mitchell AP, Cutler JE. Yeast wall protein 1 of *Candida albicans*. *Microbiology* 2005; **151**: 1631–44.
12 Blankenship JR, Mitchell AP. How to build a biofilm: a fungal perspective. *Curr Opin Microbiol* 2006; **9**: 588–94.
13 Morris DP. Bacterial biofilm in upper respiratory tract infections. *Curr Infect Dis Rep* 2007; **9**: 186–92.
14 Gilbert P, Das J, Foley I. Biofilm susceptibility to antimicrobials. *Adv Dent Res* 1997; **11**: 160–7.
15 Hawser SP, Douglas LJ. Resistance of *Candida albicans* biofilms to antifungal agents *in vitro*. *Antimicrob Agents Chemother* 1995; **39**: 2128–31.
16 Lewis RE, Kontoyiannis DP, Darouiche RO, Raad II, Prince RA. Antifungal activity of amphotericin B, fluconazole, and voriconazole in an *in vitro* model of *Candida* catheter-related bloodstream infection. *Antimicrob Agents Chemother* 2002; **46**: 3499–505.
17 Lamfon H, Porter SR, McCullough M, Pratten J. Susceptibility of *Candida albicans* biofilms grown in a constant depth film fermentor to chlorhexidine, fluconazole and miconazole: a longitudinal study. *J Antimicrob Chemother* 2004; **53**: 383–5.
18 Ramage G, Vande Walle K, Wickes BL, López-Ribot JL. Standardized method for *in vitro* antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother* 2001; **45**: 2475–9.
19 Ramage G, VandeWalle K, Bachmann SP, Wickes BL, López-Ribot JL. *In vitro* pharmacodynamic properties of three antifungal agents against preformed *Candida albicans* biofilms determined by time-kill studies. *Antimicrob Agents Chemother* 2002; **46**: 3634–6.
20 Baillie GS, Douglas LJ. Matrix polymers of *Candida biofilms* and their possible role in biofilm resistance to antifungal agents. *J Antimicrob Chemother* 2000; **46**: 397–403.
21 Kumamoto CA. A contact-activated kinase signals *Candida albicans* invasive growth and biofilm development. *Proc Natl Acad Sci USA* 2005; **102**: 5576–81.
22 Mateus C, Crow SA Jr, Ahearn DG. Adherence of *Candida albicans* to silicone induces immediate enhanced tolerance to fluconazole. *Antimicrob Agents Chemother* 2004; **48**: 3358–66.
23 Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA. Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect Immun* 2003; **71**: 4333–40.
24 García-Sánchez S, Aubert S, Iraqui I, Janbon G, Ghigo JM, d'Enfert C. *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryot Cell* 2004; **3**: 536–45.
25 Morschhäuser J. The genetic basis of fluconazole resistance development in *Candida albicans*. *Biochim Biophys Acta* 2002; **1587**: 240–8.
26 Khot PD, Suci PA, Miller RL, Nelson RD, Tyler BJ. A small subpopulation of blastospores in *Candida albicans* biofilms exhibit resistance to amphotericin B associated with differential regulation of ergosterol and beta-1,6-glucan pathway genes. *Antimicrob Agents Chemother* 2006; **50**: 3708–16.
27 Henry KW, Nickels JT, Edlind TD. Upregulation of *ERG* genes in *Candida* species by azoles and other sterol biosynthesis inhibitors. *Antimicrob Agents Chemother* 2000; **44**: 2693–700.
28 Kelly SL, Lamb DC, Kelly DE *et al.* Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol delta5,6-desaturation. *FEBS Lett* 1997; **400**: 80–82.
29 Bloch KE. Sterol structure and membrane function. *CRC Crit Rev Biochem* 1983; **14**: 47–92.
30 Ramage G, Bachmann S, Patterson TF, Wickes BL, López-Ribot JL. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *J Antimicrob Chemother* 2002; **49**: 973–80.
31 Yeater KM, Chandra J, Cheng G *et al.* Temporal analysis of *C. albicans* gene expression during biofilm development. *Microbiology* 2007; **153**: 2373–85.
32 Melkusová S, Bujdáková H, Volleková A, Myoken Y, Mikami Y. The efficiency of the benzothiazole APB, the echinocandin micafungin, and amphotericin B in fluconazole-resistant *Candida albicans* and *Candida dubliniensis*. *Pharmazie* 2004; **59**: 573–4.
33 Fonzi WA, Irwin MY. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* 1993; **134**: 717–28.
34 National Committee for Clinical Laboratory Standards. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts–Second Edition: Approved Standard M27-A2*. Wayne, Pa, USA: NCCLS, 2002.
35 Li X, Yan Z, Xu J. Quantitative variation of biofilms among strains in natural populations of *Candida albicans*. *Microbiology* 2003; **149**: 353–62.
36 Moran GP, Pinjon E, Coleman DC, Sullivan DJ. Analysis of drug resistance in pathogenic fungi. In: Kavanagh K (ed.), *Medical Mycology*. UK: Wiley, Bognor Regis, 2006: 108–10.
37 Xu J, Ramos AR, Vilgalys R, Mitchell TG. Clonal and spontaneous origins of fluconazole resistance in *Candida albicans*. *J Clin Microbiol* 2000; **38**: 1214–20.
38 Pinjon E, Moran GP, Jackson CJ *et al.* Molecular mechanisms of itraconazole resistance in *Candida dubliniensis*. *Antimicrob Agents Chemother* 2003; **47**: 2424–37.
39 Stokes C, Moran GP, Spiering MJ, Cole GT, Coleman DC, Sullivan DJ. Lower filamentation rates of *Candida dubliniensis* contribute to its lower virulence in comparison with *Candida albicans*. *Fungal Genet Biol* 2007; **44**: 920–31.
40 Maneu V, Cervera AM, Martínez JP, Gozalbo D. Molecular cloning and characterization of a *Candida albicans* gene (*EFB1*) coding for the elongation factor EF-1 beta. *FEMS Microbiol Lett* 1996; **145**: 157–62.

41 Moran GP, Maccallum DM, Spiering MJ, Coleman DC, Sullivan DJ. Differential regulation of the transcriptional repressor *NRG1* accounts for altered host-cell interactions in *Candida albicans* and *Candida dubliniensis*. *Mol Microbiol* 2007; **66**: 915–29.
42 Green CB, Zhao X, Yeater KM, Hoyer LL. Construction and real-time RT-PCR validation of *Candida albicans PALS-GFP* reporter strains and their use in flow cytometry analysis of *ALS* gene expression in budding and filamenting cells. *Microbiology* 2005; **151**: 1051–60.
43 Hawser S. Comparisons of the susceptibilities of planktonic and adherent *Candida albicans* to antifungal agents: a modified XTT tetrazolium assay using synchronised *C. albicans* cells. *J Med Vet Mycol* 1996; **34**: 149–52.
44 Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 2002; **15**: 167–93.
45 Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 2001; **9**: 34–39.
46 Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol* 2002; **292**: 107–13.
47 Nobile CJ, Nett JE, Andes DR, Mitchell AP. Function of *Candida albicans* adhesin Hwp1 in biofilm formation. Eukaryot. *Cell* 2006; **5**: 1604–10.
48 Bruzual I, Riggle P, Hadley S, Kumamoto CA. Biofilm formation by fluconazole-resistant *Candida albicans* strains is inhibited by fluconazole. *J Antimicrob Chemother* 2007; **59**: 441–50.
49 Andes D, Nett J, Oschel P, Albrecht R, Marchillo K, Pitula A. Development and characterization of an *in vivo* central venous catheter *Candida albicans* biofilm model. *Infect Immun* 2004; **72**: 6023–31.
50 Wirsching S, Moran GP, Sullivan DJ, Coleman DC, Morschhäuser J. *MDR1*-mediated drug resistance in *Candida dubliniensis*. *Antimicrob Agents Chemother* 2001; **45**: 3416–21.
51 Sullivan DJ, Moran GP, Pinjon E *et al.* Comparison of the epidemiology, drug resistance mechanisms, and virulence of *Candida dubliniensis* and *Candida albicans*. *FEMS Yeast Res* 2004; **4**: 369–76.
52 Perea S, López-Ribot JL, Wickes BL *et al.* Molecular mechanisms of fluconazole resistance in *Candida dubliniensis* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. *Antimicrob Agents Chemother* 2002; **46**: 1695–703.
53 Löffler J, Einsele H, Hebart H, Schumacher U, Hrastnik C, Daum G. Phospholipid and sterol analysis of plasma membranes of azole-resistant *Candida albicans* strains. *FEMS Microbiol Lett* 2000; **185**: 59–63.
54 Barker KS, Crisp S, Wiederhold N *et al.* Genome-wide expression profiling reveals genes associated with amphotericin B and fluconazole resistance in experimentally induced antifungal resistant isolates of *Candida albicans*. *J Antimicrob Chemother* 2004; **54**: 376–85.
55 Geber A, Hitchcock CA, Swartz JE *et al.* Deletion of the *Candida glabrata ERG3* and *ERG11* genes: effect on cell viability, cell growth, sterol composition, and antifungal susceptibility. *Antimicrob Agents Chemother* 1995; **39**: 2708–17.
56 White TC, Silver PM. Regulation of sterol metabolism in *Candida albicans* by the *UPC2* gene. *Biochem Soc Trans* 2005; **33**: 1215–8.
57 Shobayashi M, Mitsueda S, Ago M, Fujii T, Iwashita K, Iefuji H. Effects of culture conditions on ergosterol biosynthesis by *Saccharomyces cerevisiae*. *Biosci Biotechnol Biochem* 2005; **69**: 2381–8.